Molecular weight determination of Crustacean hyperglycemic hormone in the hemolymph of the blue swimmer crab, Portunus pelagicus

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***Abstract*—Hemolymph protein levels were analysed through the molting stages of P. pelagicus. A higher level of 333.63□1.50 □g/□l was observed in intermolt C stage, confirming that the C stage is metabolically active than other stages. Thus, intermolt crabs were chosen for the molecular weight determination of hemolymph proteins. SDS-PAGE and western immunoblotting studies with anti-Carcinus CHH was carried out to determine the molecular weight of CHH-like proteins. Electrophoretic separation revealed proteins in the molecular weight range of 60 kDa to 80 kDa. Immunodetectable CHH-like proteins with molecular weight of ~74 kDa was identified by western blotting. These results confirm the presence of CHH-like protein in the hemolymph of P. pelagicus.**

***Index Terms*—SDS-PAGE, Denovo sequencing, hemolymph protein, western immunoblotting**

I. INTRODUCTION

The internal secretion system of decapod crustacean consists of classical epithelial type endocrine glands and endocrine structures of neural origin, the neurosecretory cells and neurohaemal organs (Reddy and Ramamurthi, 1999). The neuroendocrine centres of crustaceans are distributed and situated in the eyestalk, subesophageal, thoracic and abdominal ganglia. The different neurohemal structures are provided by various groups of neuroendocrine cells, the X-organ–sinus gland system (XO–SG), the postcommisural and pericardial organs which are innervated by perikarya in the head and thoracic ganglion, respectively. This neuroendocrine system complex produces different kinds of neuropeptides that belong to two main functionally different families. These neuropeptides are transported along axons, to be stored in the sinus gland and released from there later (Fanjul-Moles, 2006).

Since the last two decades, numerous neuropeptides have been discovered in crustacean neuronal tissues using biological mass spectrometry (MS), which provides high speed, great sensitivity and chemical specificity (Li *et al.,* 2003; Ma *et al.,* 2008). However, compared to the widespread study of neuropeptides using tissue sources, reports on peptide analysis of hemolymph have been quite scarce, which is in part due to the significant analytical challenges for peptide detection in these complex biological fluids. For example, hemolymph contains a large number of abundant proteins including haemocyanin, cryptocyanin, plasma coagulogen, heteroagglutinins, and vitellogenins, among others (Horn and Kerr, 1963; Durliat, 1983). Chen *et al.* (2009) have evaluated three different neuropeptide protocols and developed MALDI MS profiling of hemolymph form *Cancer borealis.* A multifaceted mass spectrometric investigation of neuropeptide changes has been done in the atlantic blue crab, *Callinectes sapidus,* in response to low pH stress (Liu *et al.,* 2019).

Crustacean hyperglycemic hormone is a vital hormone both along development and during all the life cycle of the animal. The different CHH isoforms play multiple roles in decapods; they play a central role in carbohydrate metabolism, but also apply an inhibitory effect on molt and reproduction, and on osmoregulatory functions. Regarding carbohydrate regulation, this hormone induces hyperglycemia and hyperlipidemia in the hemolymph, playing a crucial role in providing glucose and lipids to meet the energy requirements of the organs and tissues of decapods (Kummer and Keller, 1993).

Instabilities of CHH secretion seem to be regulated by variations in central neuromodulation due to environmental and/or endogenous impacts. There is evidence that serotonin (5-HT), dopamine, octopamine and opioid-like peptides, such as leu-enkephalin, antagonistically modulate the secretory activity of the CHH perikarya in the XO–SG. Many authors have reported the effect of several neurotransmitters and neuropeptides on CHH release. Serotonin is a renowned neurotransmitter in crustaceans in its relationship with glycemia (Bauchau and Mengeot, 1966) and its levels have been measured in the nervous systems of various crustacean species (Laxmyr, 1984; Fingerman, 1995; Rodriguez-Sosa *et al.*, 1997; Castanon-Cervantes *et al.*, 1999; Kravitz, 2000). Additionally, 5-HT injection produced a release of CHH from the SG axon terminals in some species of crustaceans that caused hyperglycemia (Strolenberg and Van Herp, 1977). Lee *et al.* (2001) confirmed the role of this monoamine in mediating the release of CHH. Dopamine, which is present in the crustacean nervous system (Fingerman, 1995) produces hyperglycemia in the crab species, *C. maenas* (Luschen *et al.*, 1993). Webster et al. (2012) have provided an updated review on the historical development of the field of crustacean eyestalk hormones, their role in endocrine regulation, sites of expression, mode of action and target second messenger mechanisms.

Purification of CHH has been carried out in *Palaemon serratus* from total eyestalk extracts or from extirpated sinus glands, witnessing the presence of 3 different forms of hyperglycemic active fractions expressed by their electrophoretic mobility and molecular weight (Van Wormhoudt *et al*., 1984). The primary structure of CHH from the Mexican crayfish, *Procambarus bouvieri* has been elucidated by enzymatic digestion, manual Edman degradation and mass spectrometry (Aguilar *et al.,* 1995). The CHH-I and CHH-II of *P. bouvieri* have been found to have identical sequences and elicit levels of hyperglycemia that are not distinguishable. A novel CHH-like peptide (PO-CHH) and two CHH-precursor-related peptides (PO-CPRP) has been identified immunochemically and by peptide chemistry in the shore crab, *Carcinus maenas* (Dircksen et al., 2001). Tsutsui *et al.* (2016) have unravelled the crystal structure of CHH precursor suggesting structural variety in the C-terminal regions of CHH superfamily members in crustacean species.

The present study was putforth to quantify the hemolymph proteins through the molting stages, identify the hemolymph proteins by denovo sequencing and to determine the molecular weight of CHH by SDS PAGE and Western immune blotting in the hemolymph of *P. pelagicus.*

II. MATERIAL AND METHODS

2.1 Collection and maintenance of crabs

Healthy adult blue swimmer crabs, *Portunus pelagicus,* were caught from the Thondi Coast, Thondi ($9^{O}45'N$ $79^{O}04'E$) with the carapace length of 10 ± 1 cm and 80±5 g wet weight. The crabs were cautiously transported to the laboratory in aerated plastic troughs and introduced into the tank containing pre aerated filtered sea water and acclimatized for a week in tanks containing about 34±2 ppt salinity and at a temperature of (28°C). During the period, the crabs were fed with oyster meat (*Crassostrea madrasensis*) meat twice a day. The unconsumed meat and other debris particles were removed by siphoning. The water was removed and fresh sea water was introduced daily.

2.2 Molecular weight determination of CHH by SDS-PAGE

Estimation of total protein

Total protein present in the hemolymph of *P. pelagicus* was estimated following standard procedures. Hemolymph (600 µl) was mixed with 400 µl of 0.85% NaCl solution. Four ml of Biuret reagent was added to each tube (Standard and Sample) and incubated for 30 min at room temperature. After incubation, the colour developed was measured spectrophotometrically at 546 nm (Labomed Inc., USA).

SDS-PAGE (Sodium dodecyl sulphate – Polyacrylamide Gel Electrophoresis)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis is a most commonly used laboratory technique to separate different proteins based on their molecular mass. This most widely used technique was developed by Laemmli (1970).

One millilitre of cell free hemolymph was mixed with 1ml sample buffer and tubes were placed in a water bath at 95 °C for 5 min. Sample was cooled to room

temperature before loading onto the wells. Polypeptides were separated on 10% denaturing polyacrylamide slab gels and were electrophoresed by a constant 80-100 V, until the bromophenol blue marker was about 0.5 cm from the edge of the gel. Gels were stained with Coomassie brilliant blue R-250 and after incubation the gel was destained and visualized under gel documentation unit. Unstained gel was used for western immunoblotting.

2.3 Western Immunoblotting

Western blotting or immunoblotting is the most widely used technique in protein analysis. This technique detects specific antigen by the aid of an antibody (Towbin *et al*., 1979). The specificity of the antibody-antigen interaction enables a target protein to be identified in the midst of a complex protein mixture. Western blotting can produce qualitative and semi-quantitative data about the protein of interest.

The first step in immunoblotting involves the separation of proteins using electrophoresis. After electrophoresis, the gel was carefully removed from the glass plate and stacking gel was gently cut out from resolving gel. Resolving gel was equilibrated with tank buffer for 15 minutes. During this period, the electro blotting sandwich was prepared. A plastic pad was placed in tray containing transfer buffer, a sponge pad was placed on plastic pad and four sheets of filter paper were placed on the sponge pad (all were immersed in transfer buffer). The gel was now gently placed on top of the filter paper and PVDF membrane was placed carefully on the gel (avoiding air bubbles). The plastic pads were screwed and hand tightened and this sandwich was carefully placed in the tank containing transfer buffer.

Electro transfer was set to 30 V and transfer was complete by 45 min. After completion of electro blotting, the gel and membrane were gently transfer to temporary staining solution Ponceau S to visualize the bands. Membrane was then destained with water and washed 3 times with washing buffer, then the membrane was immersed in blocking solution for 2 h to block the nonspecific sites. The blocking membrane was washed 3 times with washing buffer and the membrane was incubated with primary antibody (1:10000, anti-*Carcinus maenas*-CHH) overnight at 4 °C. The membrane was then incubated in secondary antibody (1:500, HRP conjugated anti-rabbit IgG) for 2 h at room temperature. Membrane was then washed to remove unbound antibodies and transferred to petri dish containing the developer solution, DAB. The reaction was terminated using double distilled water after the development of adequate color intensity.

2.4 *De Novo* sequencing

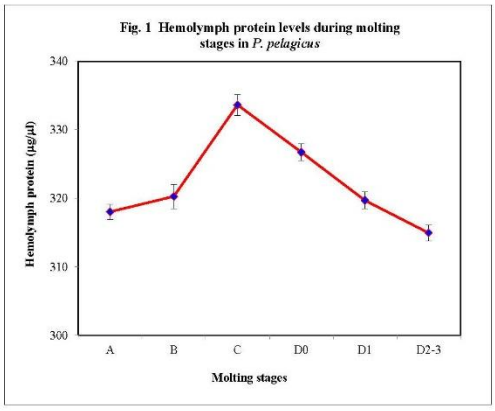
Samples were prepared for mass spectrometric analysis by using protocol of Shevchenko *et al.* (2006). Gel bands were carefully excised by using clean scalpel and cut into cubes (1 mm). Gel pieces were then transferred into centrifuge tube and 100µl of destaining solution was added. After the completion of incubation for 30 min. tubes were centrifuged at 5000 rpm for 1 min. The supernatant was decanted carefully and 500µl of acetonitrile was added and incubated at room temperature until the gel becomes white and shrunken. Gel pieces were dried in vacuum centrifuge and ice cold trypsin buffer was added into the tubes contains dried gel pieces. The tubes were incubated for 90 min to saturate them with trypsin and then 10 – 20 µl of ammonium bicarbonate buffer was added to keep the gel wet during the enzymatic digestion. Tubes were gently placed in a thermostat and incubated at 37 °C overnight. Tubes were cooled down to room temperature and centrifuged at 10000 rpm for 1 min to precipitate the gel pieces. Now, supernatant containing the peptide materials was gently aspirated (approximately 50 µl) for mass spectrometry analysis. Samples were analysed by LC-MS on an LTQ-FT (Thermo LTQ Orbitrap Discovery, Thermo Fisher Scientific Inc., USA) instrument using two fragmentation techniques, CID and CAD (y and b ions). Data were analysed by using PEAKS 7.0 search engine software. Instrument parameters: - Ion Source: ESI (nano-spray); Fragmentation Mode: CID, CAD (y and b ions); MS Scan Mode: FT-ICR/Orbitrap; MS/MS Scan Mode: Linear Ion Trap.

III. RESULTS

3.1 *Estimation of total protein*

Increase in the hemolymph proteins was witnessed during intermolt stage C (333.63□1.50 □g/ml). A gradual decrease to 314.95□1.16 □g/ml was observed thereafter till late premolt stage D2-3 followed by a steady increase in its level during postmolt stage B (320.26□1.75 □g/ml) (Fig. 1). The increase in the level

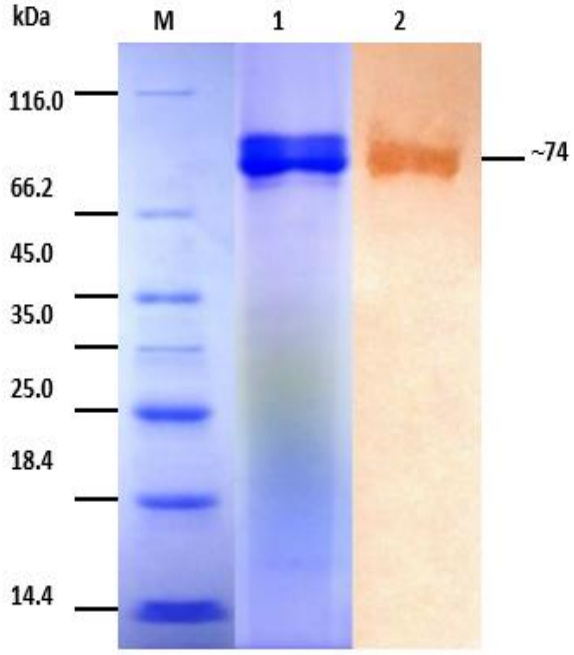
of hemolymph proteins during intermolt stage may be due to the internal tissue growth and the metabolic activities that take place during this stage. The premolt stages D0 to D2-3 are characterized by epidermal retraction and slowing down of the metabolic activities thereby resulting in decrease in the level of hemolymph proteins till apolysis. Steady increase in the level of hemolymph proteins during postmolt stages A and B could be due to the initial growth and development of the new epidermis. Thus, for studies on molecular weight determination, hemolymph samples were taken only from intermolt crabs and for further CHH experimentation, only intermolt C stage crabs were chosen.



3.2 Identification and molecular weight determination of CHH by SDS PAGE and Western immunoblotting

The processed cell free hemolymph samples were subjected to electrophoresis to separate the hemolymph proteins. Broad range molecular weight marker was run in separate lane along with the sample and the protein bands were stained with Coomassie Brilliant Blue R-250. SDS-PAGE revealed hemolymph proteins in the molecular weight range of 66 kDa to 80 kDa. Furthermore, western immunoblotting studies showed immunodetectable CHH-like proteins with molecular weight ~74 kDa (Fig. 2). Peptide spectrum matches revealed as many as 25 peptides in the hemolymph of *P. pelagicus* on mass spectrometry and peptide sequencing analysis of the gel bands (Table 1). The peptides observed in the hemolymph were cryptocyanin, hemocyanin, tropomyosin, cuticle proteins, insulin-like androgenic gland factor.

Fig. 2 Molecular weight determination of CHHby SDS-PAGE and Western Immunoblotting



M - Molecular Wight Marker 1 - SDS PAGE (10%) 2 - Western Immunoblotting

Table 1 List of proteins detected by peptide sequencing and mass spectrometric analysis in the hemolymph of *P. pelagicus*

Protein Group	Protein ID	Accession	-10lgP	Coverage (%)	#Peptides	#Unique	PTM	Avg. Mass	Description [*P.*
2	1	gi|121484232|gb|ABM54471.1|	342.43	77	53	53	Y	50097	cryptocyanin 1,
3	2	gi|123316176|gb|ABM74407.1|	292.72	75	34	23	Y	35639	hemocyanin,
1	3	gi|123316186|gb|ABM74408.1|	284.18	61	28	26	Y	32939	hemocyanin
5	6	gi|123316161|gb|ABM74405.1|	256.61	52	23	20	Y	26313	hemocyanin su 3,
4	5	gi|123316148|gb|ABM74404.1|	251.57	70	22	22	Y	25421	hemocyanin su 1,

6	4	gi|123316165|gb|ABM74406.1|	249.30	53	20	10	Y	27969	hemocyanin su 4,
13	12	gi|448278534|gb|AGE44125.1|	38.61	3	2	2	N	32809	tropomyosin
17	34	gi|698378244|gb|AIT97723.1|	26.08	26	2	2	Y	18221	glyceraldehyde 3-
45	16	gi|899684290|gb|AKR18000.1|	25.65	3	1	1	N	63808	NADH
45	15	gi|749111978|ref|YP_009116116.1|	25.65	3	1	1	N	63808	NADH
45	14	gi|745748908|gb|AJD22390.1|	25.65	3	1	1	N	63808	NADH
45	13	gi|937957491|gb|ALJ01875.1|	25.65	3	1	1	N	63808	NADH
19	11	gi|121484195|gb|ABM54458.1|	25.17	7	1	0	Y	25409	cuticle protein
33	26	gi|121484201|gb|ABM54460.1|	25.17	9	1	0	Y	19626	cuticle protein
44	25	gi|121484199|gb|ABM54459.1|	25.17	7	1	0	Y	23979	cuticle protein
91	72	gi|300915863|gb|ADK46885.1|	23.32	16	1	1	Y	19679	insulin-like
48	60	gi|189310188|emb|CAP20051.1|	22.38	6	1	1	Y	40192	transposase
48	59	gi|189310193|emb|CAP20054.1|	22.38	6	1	1	Y	40053	transposase
93	90	gi|110431786|gb|ABB22040.1|	20.52	5	1	1	Y	25990	putative farnesoic
93	89	gi|110431772|gb|AAZ40196.1|	20.52	5	1	1	Y	25990	putative farnesoic
93	92	gi|110431788|gb|ABB22041.1|	20.52	5	1	1	Y	26276	putative farnesoic
93	91	gi|110431774|gb|AAZ40197.1|	20.52	5	1	1	Y	26276	putative farnesoic
93	94	gi|78127420|gb|ABB22042.1|	20.52	5	1	1	Y	26435	putative farnesoic
93	93	gi|110431776|gb|AAZ40198.1|	20.52	5	1	1	Y	26435	putative farnesoic
63	36	gi|122003901|gb|ABM65760.1|	20.23	5	1	1	N	14746	chymotrypsin-like

IV. DISCUSSION

The levels of proteins, carbohydrates and lipids are an expression of an animal's adaptive characteristics and its strategies for adaptation. Many biotic (e.g., maturation, reproduction and food availability) and abiotic factors (e.g., photoperiod, temperature, pH and oxygen in water) can strongly affect the biochemistry and physiology of decapod crustaceans (Company and Sardà, 1998; Rosa and Nunes, 2003 a, b; Vinagre *et al.,* 2007). Arthropod growth patterns characterized by molt cycles present some trade-offs such as the need for variable muscle atrophy and restoration to accommodate the body inside the new slightly bigger exoskeleton.

The molt cycle drives extensive behavioural, integumentary, physiological, and biochemical changes in crustaceans. Besides its role in digestion, the digestive gland or hepatopancreas actively participates in the molt cycle, being the major site for storage glycogen, fats, and calcium during premolt and thus, in the mobilization of these reserves when needed in subsequent molt stages. Among fatty acids, the 16:1 n−7 and 18:1n−9 fatty acids are important as energy source for growth, molt and during starvation for various crustacean species, such as the kuruma shrimp *Penaeus japonicus* (Teshima *et al.,* 1977), the giant freshwater prawn *Macrobrachium rosenbergii* (Sithigorngul *et al.,* 1997) and the Chinese mitten crab, *E. sinensis* (Wen *et al.,* 2006).

In the present study, the proximate composition of biochemical constituents in the molting stages of *P. pelagicus* revealed high levels of protein during the intermolt stage, which could be due to internal tissue growth (Passano,1960 a,b), and considerably low in postmolt, premolt and molt. As the growth starts to occur during the stages A-B (postmolt), the rate of growth decreases in the premolt stage D and the rate of feeding also decreases (Freeman and Perry, 1985). In large clawed crustaceans, elevated protein degradation has been associated with molt-induced muscle atrophy during early premolt, and this phenomenon is necessary to allow muscle withdrawal from the exoskeleton at molt (Shean and Mykles, 1995). In post molt stage A-B, the percentage of protein, calcium and chitin was observed to be high by Vigh and Dendinger (1982). In the intermolt stage C, a metabolically and morphogically stable stage, the

formation of the internal cone at the base of the seta takes place (Drach, 1944; Scheer, 1960; Kamiguchi, 1968; Peebles, 1977). The growth of muscles takes place during premolt stage, which has been confirmed by the higher protein levels (Alvarez- Fernadez *et al.,* 2005). According to Adiyodi and Adiyodi (1970) and Quakenbush and Herrnkind (1981), the reproductive activity occurs in both male and female during this stage as the nutrient stores are sequestered in this stage. The males mate during this stage and the females are said to carry eggs during this stage. No change in the cuticle occurs during this stage. After ecdysis, proteins in the claw muscle are rapidly synthesized to replace the myofibrillar proteins degraded during the atrophy (West, 1997).

Separation of the hemolymph proteins from *P. pelagicus* was accomplished electrophoretically based on their molecular weight. The proteins thus separated where subjected to Western immunoblot analysis to identify the molecular weight and confirm the presence of CHH. SDS-PAGE displayed protein bands in the molecular weight of 66 – 88 kDa. The result of the western blotting studies showed ~74 kDa immunodetectable protein band on SDS PAGE and with high cross reactivity to anti- *Carcinus maenas*-CHH.

Kallen *et al.* (1990) reported most of the detectable proteins in the hemolymph of *Orconectes limosus* varying in molecular weight between 56 to 150 kDa. In addition, they investigated that immunochemically and biologically most active gel filtration fraction by using SDS-PAGE and immunoblotting, finally found molecular weight of that immunodetectable proteins with molecular weights around 56-150 kDa. These gel fractions showed hyperglycemic activity in crayfish. The main fraction out of the three different forms of hyperglycemic active fractions purified from the total eyestalk extracts or extirpated sinus glands of *P. serratus,* had a molecular weight of around 8000 Da but the small peptides had molecular weights around 2000 Da (Van Wormhoudt *et al*., 1984). Aguilar *et al.* (1995) identified CHH-I as a 72 residue peptide with a molecular mass of 8388 Da, six cysteine residues forming three disulphide bridges that connect residues 7-43, 23-39 and 26-52 from *P. bouvieri*. The peptide also had blocked N- and C- termini, and lacks tryptophan, histidine and methionine. Dircksen *et al.* (2001) have identified PO-CHH, a 73 amino acid peptide with a free C-terminus and a molecular weight of 8630 Da, in the *C. maenas.*

The pattern of this electrophoresis corresponds to the electrophoretic behaviour of crustacean hemocyanins as described by Markl *et al.* (1979). Immunoblotting results of brain extract of *S. dichotomus* showed positive immunoreactivity to CHH (Nithya and Munuswamy, 2002). Our results of denovo sequencing also paralleled with Kallen *et al.* (1990) research findings. Five neuropeptide families such as RFamide, allatostatin, orcokinin, tachykinin related peptide, crustacean cardioactive peptide and several other neuropeptides have been detected by *de novo* sequencing in *C. borealis* (Chen *et al*., 2009). Liu *et al.* (2019) have identified two novel peptide sequences in the hemolymph of *C. sapidus* that are involved in stress regulation.

V. CONCLUSION

Variations in the level of proteins were observed in the hemolymph through the molt stages of *P. pelagicus,* with a higher level witnessed during intermolt C stage. A clear separation of hemolymph proteins was observed by SDS-PAGE studies, which on further analysis by western immunoblotting revealed CHH-like immunoreactivity at a molecular weight of ~74 kDa. With the knowledge, future work will focus on studying the functional roles and modulation mechanism of CHH and other hemolymph proteins.

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